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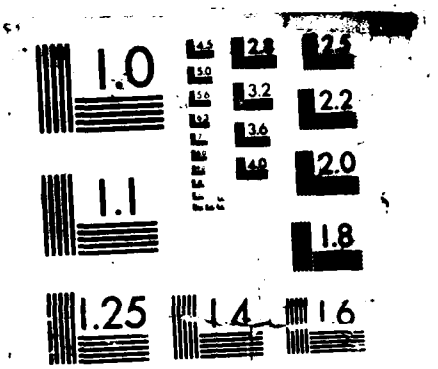

MICROCOPY RESOLUTION TEST CHART

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Development of a DNA-Based Method for Distinguishing the Malaria Vectors, Anopheles gambiae from Anopheles arabiensis

Annual Report

Victoria Finnerty, Ph.D.

June 1987

Submitted to:

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The Anopheles gambiae complex includes six morphologically identical species, two of which (A. gambiae and A. arabiensis) are the primary African malaria vectors today. [illegible] more of the species are commonly sympatric, epidemiological studies to determine the [illegible] ment of each in malaria transmission have been difficult. This report [illegible] to develop a DNA probe to distinguish A. gambiae from A. arabiensis. [illegible] ment of rDNA from A. gambiae which displays an RFLP when the two species are [illegible] them analysis. Thus far the probe has proven to be extremely sensitive [illegible] even with short exposures to [illegible] single adult mosquitoes [illegible] sexes. Larvae and pupae are similarly easy to [illegible]. [illegible] room temperature [illegible] months [illegible]. We have [illegible] probe method [illegible] used for [illegible] during DNA extraction.

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Development of a DNA-Based Method for Distinguishing the Malaria Vectors, Anopheles gambiae from Anopheles arabiensis

Annual Report

[illegible], Ph.D.

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Contract No. DAMD17-85-C 5184

SUMMARY

The Anopheles gambiae complex includes six morphologically identical species, two of which (A. gambiae and A. arabiensis) are the primary African malaria vectors today. Since two or more of the species are commonly sympatric, epidemiological studies to determine the involvement of each in malaria transmission have been difficult. This report describes our efforts to develop a DNA probe to distinguish A. gambiae from A. arabiensis. The DNA probe is a fragment of rDNA from A. gambiae which displays an RFLP when the two species are compared by Southern analysis. Thus far the probe has proven to be extremely sensitive since it can be used even with short exposures to diagnose single adult mosquitoes (or parts thereof) of both sexes. Larvae and pupae are similarly easy to distinguish. Specimens kept dessicated at room temperature for as long as nine months can be scored. We have demonstrated that the DNA probe method can also be readily used on dessicated abdomens, while the thoraces have been used for sporozoite analysis. Blood meal analysis is easily done from the protein pellet obtained during DNA extraction. The DNA probe method has been directly compared to the GDH isozyme method and no exceptions were found. The DNA probe method can diagnose a number of individuals bearing rare GDH alleles which cannot be scored enzymatically. Finally, the DNA probe method, when directly compared to the cytogenetic speciating technique shows virtually complete agreement. The method has some [illegible]

FOREWORD

Studies with Recombinant DNA: The investigator has abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1983) and the Administrative Practices Supplements.

Emory University Biosafety File No. 142-85

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ANNUAL REPORT

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2. Background: Many of the major malaria vectors are members of species complexes, for instance, A. culicifacies (12), A. leucosphyrus (13), and the A. far auti sibling series (14). In these complexes, as well as in the A. gambiae complex, reliable species identification of individuals is currently tedious and difficult. Since malaria continues to represent a major world health problem, epidemiological studies with these species is crucial.

Our studies focused on two sympatric species, A. gambiae and A. arabiensis. The proposal hypothesized that the genomic DNA of these two species currently differs in ways that would permit reliable species identification. In particular, we sought to develop a species differentiating assay based upon restriction fragment length polymorphisms detected by either heterologous or species-specific probes. During our first year of work we found that certain species specific sequences would be most useful for the assay we sought to develop, and therefore our efforts have focused upon these sequences rather than heterologous probes.

3. Rationale: A substantial body of evidence argues that [illegible] between members of related species (15,16,17). Given that we expected [illegible] between A. gambiae and A. arabiensis, then such differences would provide an excellent epidemiological tool. The major advantage of a DNA based assay is the great sensitivity of Southern analysis so that [illegible] parts thereof could be examined, and [illegible] that field specimens could be assayed.

[illegible]

least 200 copies per genome arranged in a few large tandem assays (18). Therefore, the rDNA genes possess the ability to yield useful RFLPs as well as species-specific sequences, both of which would be the basis for a diagnostic assay.

4. Experiments and Results: Development of a single mosquito species assay used to distinguish A. gambiae from A. arabiensis. The strategy employed in this work was to quickly identify portion(s) of an rDNA gene in A. gambiae which were non-coding regions; i.e., the introns and spacers. Such non-coding DNA fragment(s) would then be the basis for further studies to determine whether they could reveal an RFLP in A. arabiensis DNA.

(i) Isolation of a diagnostic cloned rDNA fragment. An A. gambiae genomic library was screened with a Sciara coprophila rDNA clone (19) which contains one complete cistron. Thirty-two A. gambiae rDNA-containing phage were isolated and selected for further analysis. These clones were restricted with various enzymes and subjected to Southern analysis, in order to find nonconserved regions that might be used to reveal differences between the species. The blots were therefore probed with Sciara rDNA which is not expected to hybridize to fragments from the nonconserved regions. Restriction fragments from such regions (those not hybridizing to the Sciara probe) were then isolated from gels and used to probe genomic Southern blots of A. gambiae and A. arabiensis DNA. Clone λAgr12, shown in figure 1, was found to contain a 0.59kb EcoRI-SalI restriction fragment which consistently showed a different pattern of hybridization to A. gambiae versus A. arabiensis genomic DNA. The 0.59kb EcoRI-SalI fragment is very close to the 3' terminus of the 28S region of the mosquito rDNA cistron. Hybridization of the Sciara and Calliphora probes is very weak in this region, suggesting a low degree of conservation, yet this fragment is highly conserved among different geographic isolates of the three member species in the A. gambiae complex thus far examined. EcoRI-SalI genomic digests invariably show the 0.59kb fragment, and there is no evidence for detectable levels of intercistronic variation in either of these two restriction sites. In summary, the probe shows an unambiguous difference between members of the complex thus far examined.

(ii) [illegible] of the diagnostic fragment to single dried mosquitoes. The 0.59kb fragment was subcloned into the Bluescribe M13+ plasmid (Stratagene Cloning Systems), and this construct, pAg176, has been used to probe a large [illegible] of A. gambiae complex colonies and field isolates. Mosquito genomic DNA [illegible]

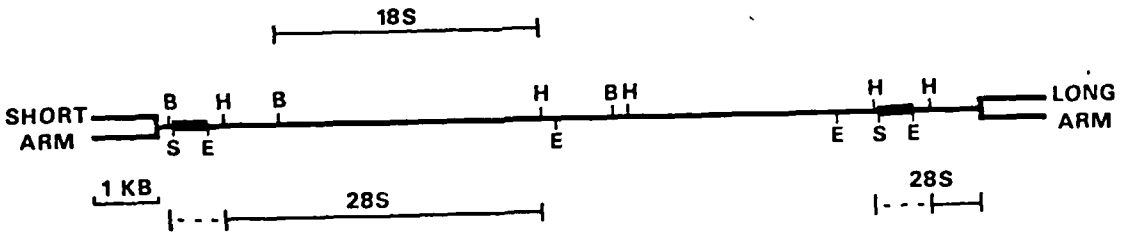

Fig. 1. λAgr12 Restriction map. The approximate locations of the 18S and 28S regions were determined by hybridization with heterologous Sciara rDNA (pBC2), kindly provided by S. Gerbi, and Calliphora rDNA (pKB-42 and pKB-33), kindly provided K. Beckingham. λAgr12 contains slightly more than 1 rDNA cistron, including the NTS. The dashed line indicates weak hybridization to the heterologous probes. The .59kb EcoRI-SalI restriction fragment which reveals a diagnostic restriction fragment length polymorphism between A. gambiae and A. arabiensis is shown as a darkened bar.

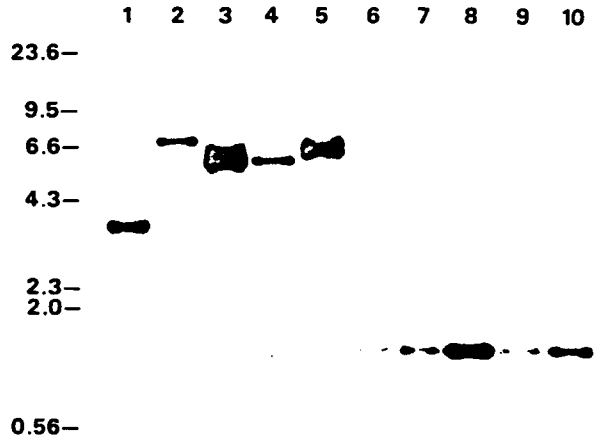

Fig. 2. Hybridization of pAgr12A to EcoRI digests of single dried female mosquitoes. Species and geographic origin of specimens are as follows: (1) A. melas (The Gambia), (2) A. arabiensis (Sudan, SENNAR colony), (3) A. arabiensis (Sudan, G/MAL colony), (4) A. arabiensis (Kenya), (5) A. arabiensis (Burkina Faso), (6) A. gambiae (Tanzania), (7) A. gambiae (Zanzibar), (8) A. gambiae (Kenya), (9) A. gambiae (Nigeria), (10) A. gambiae (The Gambia, GB colony).

tion in A. arabiensis and A. melas are probably due to inter-cistronic variation in the spacer region.

In summary, specimens dessicated by a very simple method show no evidence of DNA degradation even when stored at room temperature for as long as nine months. Moreover, in other preliminary experiments we found that other life stages such as second instar larvae and pupae (and obviously both sexes) are readily scored by the DNA probe.

(iii). Genomic location of the diagnostic probe. Organization of the rDNA cistron appears to be the same in both males and females, as judged by Southern blots of male and female DNA. However, the intensity of hybridization of pAgr12A to genomic Southern blots, as shown in figure 3, indicates that males have a smaller number of total copies, which is expected if the rDNA genes reside on the X chromosome. A. gambiae-A. arabiensis hybrid female mosquitoes reared in the laboratory contain both of the parental types of rDNA cistrons (Fig. 3). Male hybrids, on the other hand, show the cistron structure of the female parent, indicating that the rDNA genes are located primarily if not exclusively on the X chromosome. This finding directly associates the diagnostic probe with that part of the mosquito genome (the X chromosome) currently used as the basis for cytogenetic speciation.

(iv). Compatibility of the DNA probe method with the sporozoite assay and blood meal analysis. In order to determine whether the probe could be used to assay single mosquitoes for the presence of the malaria parasite, we obtained a number of field specimens which had been dessicated for at least 14 months. The mosquitoes were cut so that Dr. Collins retained the head and thorax for the sporozoite assay (21) and we tested the abdomens. The results, shown in Table 1, indicate that the diagnostic probe can readily distinguish species of only part of a dried specimen. The proportions of gambiae and arabiensis in Asembo which we found are similar to those found by other workers. Since these specimens were quite old and we did these experiments at a time when our DNA extraction procedure had not been optimized, there are an unacceptable number of unreadable individuals shown in Table 1. Since then, however, we have had few if any unreadable abdomens from the specimens so treated.

A second important consideration for a diagnostic probe is whether it is compatible with blood meal analysis. The DNA extraction [illegible] we [illegible] allows blood meal analysis: A single mosquito [illegible] homogenized in a 1.5ml plastic Eppendorf tube using a [illegible] glass pestle [illegible] 50μl .08M NaCl, .16M sucrose, .06M EDTA, .5% SDS, .1M Tris [illegible] pH 8.6. The homogenate is incubated, 65°C, 30 [illegible] 8M potassium acetate [illegible] [illegible]. After incubation, 4°C, [illegible] [illegible] supernatant is removed to a fresh tube [illegible] the pellet is saved. [illegible] 95% ethanol is added to [illegible] [illegible] After [illegible] the supernatant, the DNA [illegible] [illegible] The first potassium acetate pellet contai[illegible] [illegible] [illegible] Table 1 [illegible] [illegible] meal [illegible]

[illegible] [illegible]

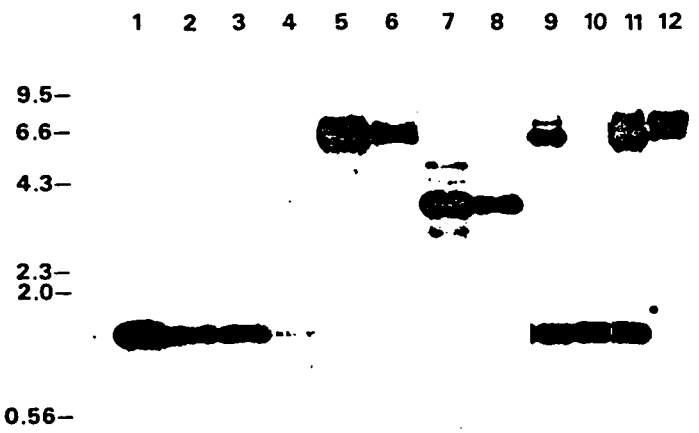

Fig. 3. Hybridization of pAGr12A to single dried male and female mosquitoes or mosquito abdomens. Lane (1) A. gambiae female, (2) A. gambiae female (blood-fed), (3) A. gambiae female (abdomen only), (4) A. gambiae male, (5) A. arabiensis female, (6) A. arabiensis male, (7) A. melas female, (8) A. melas male, (9) A. gambiae-A. arabiensis hybrid female, (10) A. gambiae-A. arabiensis hybrid male, (11) A. arabiensis-A. gambiae hybrid female, (12) A. arabiensis-A. gambiae hybrid male. Female parent is listed first for all hybrids. DNA from a single abdomen is clearly more than sufficient to make a species identification. Furthermore, the presence of a blood meal in the abdomen does not significantly reduce DNA yield. Dessicated individual pupae and larvae (all instars except the first) can also be readily speciated.

Table 1. Result of testing abdomens of A. gambia complex mosquitoes collected in Asembo area of Kenya in October 1985.

Abdomens from:	Species		DNA not readable
	A. gambia	A. arabiensis	
Plasmodium falciparum infected mosquitoes	47 (75%)	17 (27%)	8
Uninfected mosquitoes	78 (49%)	80 (51%)	19

Note: percentages are based on specimens which were identified as to species. The sporozoite assay (21) and DNA probe assay were performed in December 1986.

lected by Dr. Collins in western Kenya (Ahero, Asembo, and Gumbe) and [illegible] area (Sabaki), which are shown in figure [illegible], were used. These [illegible] fested areas where the two species are known to be sympatric. [illegible] gated, [illegible] rice-growing area [illegible] the [illegible] plain approximately [illegible] east of [illegible] Kisumu. Previous studies of the mosquito fauna of this [illegible] have shown that most of the A. gambiae complex mosquitoes breeding [illegible] biensis (22). Asembo is a farming community located [illegible] the [illegible] Victoria, approximately [illegible] west of [illegible]. [illegible] at a slightly higher elevation [illegible] Gumbe [illegible] considerably more [illegible] either Ahero or Asembo. Both A. gambiae [illegible] from these areas (22). Sabaki [illegible] north of Mombasa [illegible] A. gambiae [illegible] [illegible]

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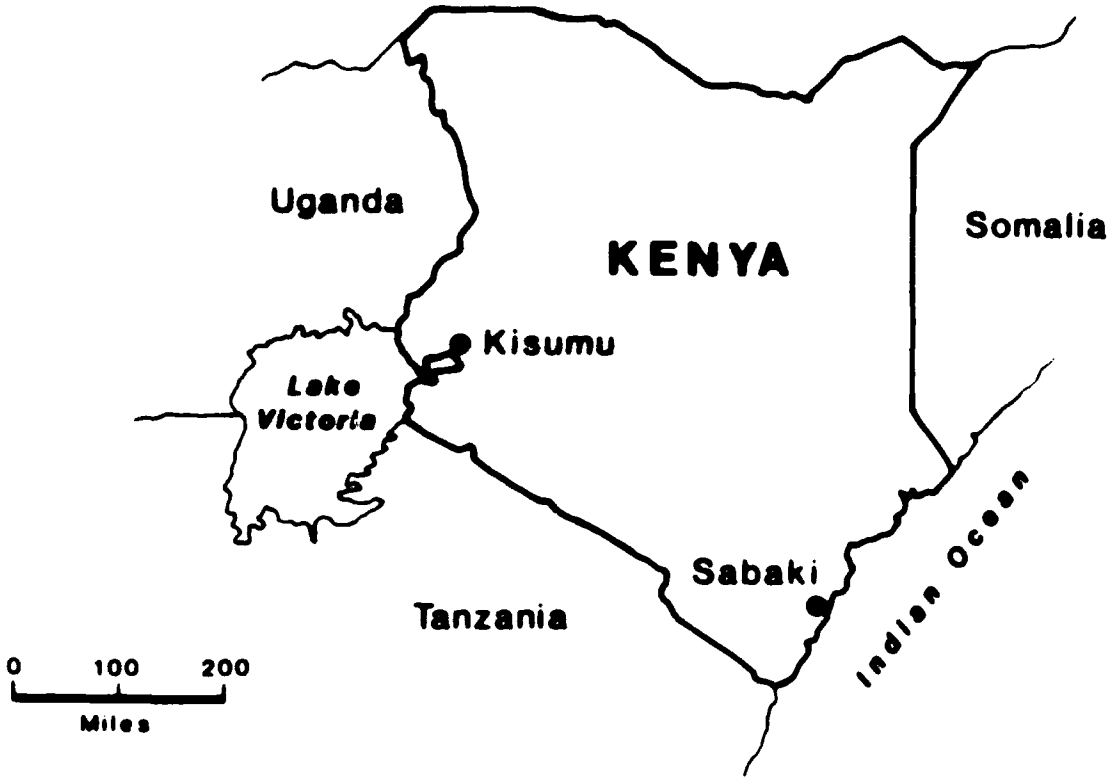

Figure 4. Map of Kenya showing locations from which specimens were obtained.

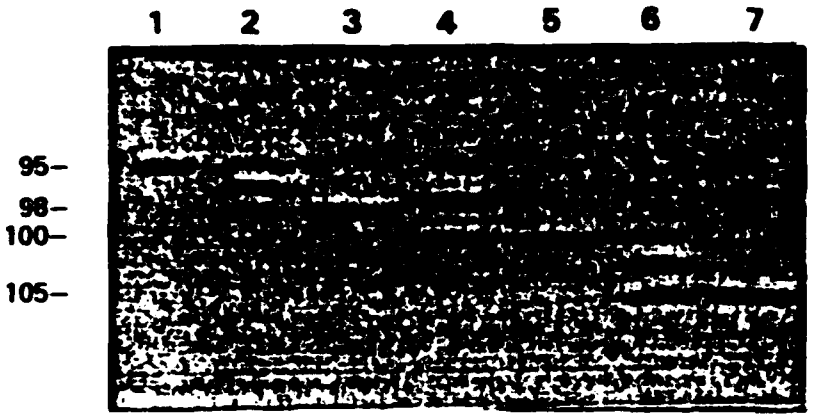

Fig 5

Octanol dehydrogenase electromorphs found in the Kenya field samples. Lane 1, A. arabiensis from the G/MAL colony; lanes 2-3 are A. arabiensis from Ahero; lane 4, is an A. arabiensis (G/MAL) x A. gambiae (G3) hybrid produced in the laboratory; lanes 5-7, are A. gambiae from the GO-66 colony established with specimens collected in Gombe.

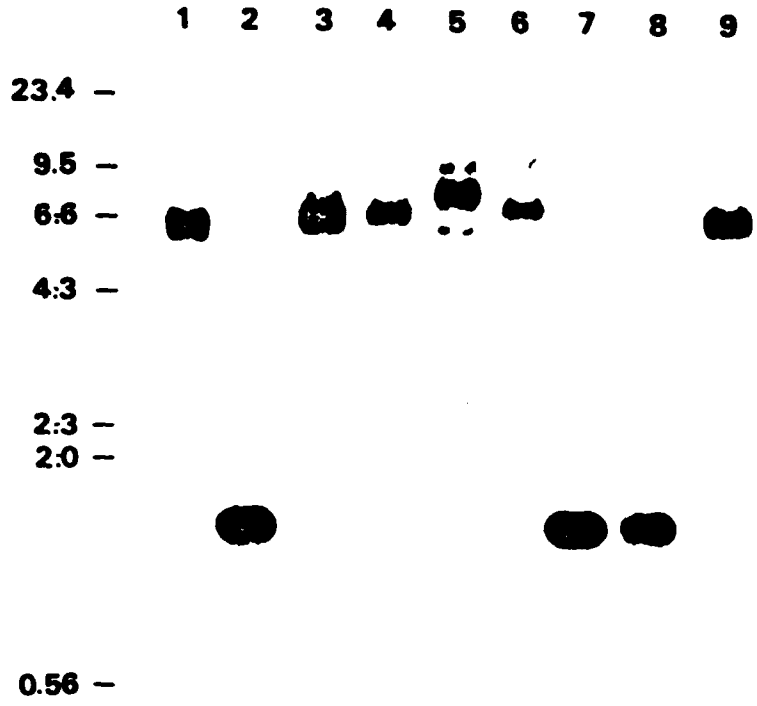

Figure 6 Hybridization of the pAgl12A probe to EcoR1 digests field-collected specimens. Lanes 1-5, individual mosquitoes from different Ahero families; lanes 6-9, individuals from Gombe families.

Table 2. DNA probe and Odh isozyme analyses of Anopheles gambiae complex mosquitoes from Kenya.[a]

Location	Probe-Checked Families		ODH Alleles Present				
	No.	Probe Result	90	95	98	100	105
Ahero	1	A. gambiae				+	
	3	A. arabiensis		+	+		
	1	A. arabiensis		+			
	1	A. arabiensis	+	+			
Asembo	1	A. gambiae				+	+
	8	A. gambiae				+	
	1	A. gambiae			+	+	
	1	A. arabiensis		+	+	+	
	1	A. arabiensis			+		
	1	A. arabiensis	+	+	+		
	4	A. arabiensis		+	+		
	3	A. arabiensis		+			
Gombe	10	A. gambiae				+	
	1	A. arabiensis		+	+	+	
	1	A. arabiensis		+		+	
	1	A. arabiensis	+	+		+	
	1	A. arabiensis		+	+		
	1	A. arabiensis	+	+			
Sabaki	86	A. gambiae				+	
	1	A. gambiae				+	+
	25	A. arabiensis		+			

[a] Results for material from Ahero, Asembo, and Gombe represent analysis of at least two mosquitoes from each family for DNA type and an additional two mosquitoes for Odh isozymes. Results for material from Sabaki represent DNA probe and Odh isozyme analyses on single mosquitoes (the abdomen being used for Odh analysis and the head-thorax portion being used for DNA typing).

alleles test as A. gambiae by DNA probe; those with ODH-95 have the A. arabiensis DNA pattern.

Specimens from the western Kenya locations were treated somewhat differently in that individuals from each isofemale family were analyzed either by DNA probe (two individuals/family) or ODH isozyme (two or three individuals/family). Of the 41 different families so analyzed, none showed any within-family variation in the DNA probe hybridization pattern. Furthermore, only the expected 1.4kb or [illegible] 8kb bands of hybridization were observed.

All the previously cited studies of ODH alleles in field specimens of A. gambiae and A. arabiensis indicate that, with near certainty, families with only the ODH-1[illegible] or ODH-1[illegible] alleles can be classified as A. gambiae and families with only the ODH-[illegible] or ODH-95 alleles are A. arabiensis. Indeed, the 20 families from Ahero, Asembo, and Gambo with the A. gambiae isozyme type show the DNA probe pattern diagnostic of A. gambiae. Also, the six families with only alleles ODH-[illegible] or ODH-95 are identified by the probe as A. arabiensis. The fifteen families with other combinations of ODH alleles cannot be assigned to species on the basis of their isozyme pattern. However, the DNA probe test of these families indicates that [illegible] of the 15 are A. arabiensis, a finding that is consistent with those of Miles [illegible] and others who have reported considerably higher frequencies of [illegible] in [illegible] populations of A. arabiensis than A. gambiae. None of the [illegible] individuals examined in this study gave a DNA probe pattern that would suggest an interspecies hybrid.

[illegible] specimens from Jabana and the [illegible] families [illegible] results [illegible] a reliable species diagnosis [illegible] agreement with those [illegible] by the DNA probe. [illegible]

Table 3. Results of testing individual field specimens by DNA probe and cytogenetic methods.

COLLECTION SITE	CHROMOSOME RESULT	DNA RESULT		
		SAME AS CHROMOSOME	DIFFERENT FROM CHROMOSOME	NOT DONE OR NOT READABLE
ZIMBABWE	A. arab. (10)	10	0	0
	A. quad. (41)	41	0	0
KENYA				
Ahero	A. arab. (30)	76	0	4
Asembo/Boi	A. arab. (28)	25	1 (A. gamb.)	2
	A. gamb. (88)	70	1 (A. arab.)	17

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